Influence of acid hydrolysis, saponification and sample clean-up on the measurement of phytosterols in dairy cattle feed using GC–MS and GC with flame ionization detection

Influence of Acid Hydrolysis, Saponification and Sample Clean-up on the Measurement of Phytosterols in Dairy Cattle Feed Using GC/MS and GC/Flame Ionization Detection

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Running title: Phytosterol Content in Dairy Cattle Feed Using GC/MS/FID

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Non-standard Abbreviations:

flame ionization detection (FID); N,O-bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1%TMCS); linearity dynamic range (LDR); certified reference materials (CRM); National Institute of Standard and Technology (NIST); secondary reference material (SRM); measurement uncertainty (MU); limit of reporting (LOR).

Keywords: phytosterols, dairy cattle feed, sample pretreatment, flame ionization detection

Abstract

The fortification of processed foods including dairy products is increasingly commonplace with phytosterols among the many compounds used to improve the nutritional value of food products. It is also increasingly common practice for some dairy cattle feeds to be fortified for their potential to increase phytosterol levels in milk. In this paper, a combined, streamlined protocol using acid hydrolysis, saponification and sample clean-up was developed to enable the rapid and reliable. The method was developed with focus on streamlining the overall technique to make it suitable for commercial laboratories, to reduce labor and consumable costs, while maintaining accuracy. A total of twelve different feed types commonly used in the dairy industry were analyzed with the highest and lowest sterol contents found in cotton seed oil and tannin with average phytosterol contents of 256 mg and <30 mg per 100 g, respectively. With a limit of reporting of 30 mg/kg for individual sterols and a correlation coefficient >0.99, the method was validated for milk to enable feeding comparison studies with respect to the total phytosterol content in raw milk.

1 Introduction

The Australian dairy industry has a net worth of over 1.5 billion dollars with up to 30% of all production exported internationally, predominately to Asia [1]. With a net share of 6% worldwide, Australian dairy companies are collectively the third largest global dairy industry. To meet production demands, farmers typically invest around 30% of the farming budget towards pasture production which can include planting, harvesting, feeding and storage. In 2014/15, there were approximately 9.7 billion liters of milk produced in Australia [1], but rising costs associated with dairy farming have led to a decline in the total number of productive farms. A growing demands for dairy supplies has required the remaining farms to increase their herd size and, over the past 30 years, numbers have increased from around 85 to the current average of 284 cows per farm [1, 2]. Herd sizes will most likely continue to expand to meet growing demands and this will subsequently increase feed requirements.

Phytosterols are plant based sterols that are the equivalent of cholesterols in animals and with more than 200 forms identified [3], these are vital structural components of plant membranes and other metabolic precursors [4, 5]. There are 5 main forms of phytosterols including free, hydroxycinnamic acid esters, steryl esters, steryl glycosides and acylated steryl glycols, with the latter known as conjugates [3]. The health benefits relating to the consumption of phytosterols are well understood with studies showing a reduction in dietary cholesterol and subsequent lower risk of cardiovascular disease [3, 6, 7]. With the status of “generally recognised as safe” granted by the United States Food and Drug Administration [6, 7], phytosterols are commonly fortified in various food products including milk, cheese, chocolate, pasta and fat spreads, among many others [3, 6, 8]. Worldwide, leading food authorities generally permit producers of products containing phytosterols to advertise health claims such as those related to a reduction in cholesterol or reduced risks of cardiovascular disease [7, 9, 10]. There have been few long-term studies into the effects of high

phytosterol consumption, however some short-term studies have demonstrated that a diet high phytosterols can impair vitamin D adsorption and reduce carotenoid levels in plasma by 15-20% [11-13].

There is an ever-increasing interest in enhancing the health benefits of various food products with a major focus on milk and other dairy products [14-16]. Such enhancements are ideally achieved naturally through changes in the feeding of dairy cows to ensure food safety, to minimize human error and subsequent over fortification or consumption. At present, there are a few reported studies investigating the influence of cattle feed on the enhancement of macro- or micro-nutrients in milk. However, these are primarily focused on products that are fortified after milk production with few related to phytosterol fortification [8, 17, 18]. As a result, there is a growing need for the rapid, streamlined, and accurate measurement of phytosterols and their conjugates in an array of matrices [19, 20].

Many past studies have used only saponification to determine phytosterol fatty esters as these are the forms commonly found in fortified food products [21-24]. However, due to the significant amount of sterol conjugates, a technique liberating all phytosterol forms including the conjugates is necessary to determine total phytosterol levels [6, 8, 25, 26]. Therefore, acid hydrolysis has been used to liberate sterol glycosides which is then followed by saponification to ensure all sterol conjugates are liberate and are free for extraction [27]. There is an increasing concern with the use of acid hydrolysis, however, since the isomerization of avenasterol and fucosterol has been observed when using this technique. Although this has led to the development of alternative means such as enzymatic treatments to avoid this complication [28-30]. In most food matrices, avenasterol and fucosterol are generally found in insignificant amounts compared to total phytosterols and therefore the use of acid hydrolysis is generally suitable [6, 26, 31].

Another disadvantage of using acid hydrolysis is the aggressive nature of the reaction that can lead to the increased extraction of non-targeted compounds. Adequate sample clean-up is therefore essential for sterol quantification in order to improve instrumentation analysis by removing potential interfering compounds. Techniques including TLC have been used in the past and although the method was effective, it was found to be time consuming and lacked the required rigor for routine testing [32, 33]. Other methods based on SPE have been reported as being more efficient and convenient techniques allowing for the purification of sterols prior to analysis [34, 35].

In our recent study, an optimized saponification method for the determination of total sterols was reported [36]. In the present paper, an extension of this method is reported for the determination of total phytosterols and their conjugates in dairy cattle feed with a view to assessing major cattle feeds commonly used in Australia. A novel extraction technique including acid hydrolysis, sample clean-up and analysis by GC/MS and GC/flame ionization detection (FID) was optimized and a range of feeds were analyzed for total phytosterol content.

2 Materials and methods

2.1 Standards and other chemicals

Sterol standards for quantification (with certified assay purities given in brackets) included: cholesterol (99%), brassicasterol (95%), campesterol (65%), stigmasterol (95%), lanosterol (93%), β-sitosterol (97%), cholestenol (95%), lathosterol (98%), fucosterol (93%), stigmastanol (95%) and demosterol (84%). A surrogate standard, 5β-cholestan-3α-ol (98%), was added at the beginning of each extraction to compensate for any degradation or loss from the extraction process, for quality assurance purposes, and as an internal standard during the data interpretation stage. All standards were purchased from Sigma Aldrich (Sydney, Australia) or Steraloids Inc. (Rhode Island, USA) and were prepared in cyclohexane with a stock concentration of 500 mg L^{-1}.

105 Solvents including cyclohexane, hexane, toluene, n-heptane, ethanol, chloroform, and methanol
106 and hydrochloric acid were purchased from Merck Australia (Melbourne, Australia) with purities
107 greater than 95%. Pyridine was obtained from Sigma Aldrich (Sydney, Australia). N,O-bis-
108 (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1%TMCS) was purchased
109 from Grace Davison (Melbourne, Australia). Deionized water was used throughout the experiments
110 and was obtained using a Millipore water purification system (Element A10).
111 The reagent 5 M potassium hydroxide (assay purity 85% from sigma Aldrich) was prepared in a
112 solution of 10:90% (v/v) water:ethanol. Different hydrochloric acid hydrolysis solutions were used: (i)
113 prepared in 100% water for cattle feed samples (4 M HCl), (ii) 8 M HCl prepared in ethanol for
114 unfortified milk, and (iii) prepared in 50% ethanol in water non-cattle feed samples (4 M HCl). The
115 solvents used for solid phase extraction included n-heptane, chloroform and a methanol:chloroform
116 mixture (20:80% (v/v)).
117 A total of 3 samples were used to represent the common matrices studied and to efficiently
118 develop and validate the methods for a large range of matrix types. These representative samples
119 included milk powder with a known cholesterol content of 13 mg/100 mL and Vega pure E with a
120 certified total phytosterols content of 6200 mg/100 g. The milk powder and Vega pure E were used to
121 represent the fatty matrices and lucerne hay was used for cattle feed samples.
122 **2.2 Sterol quantification**
123 Quantification of the phytosterols was achieved using FID with appropriate reference standards
124 and 5β-cholestan-3α-ol as the internal standard to create traditional calibration curves. Confirmation
125 of sterol identification was achieved using MS [37]. The linearity dynamic range (LDR) for the majority
126 of individual phytosterols was found to be 0.1 to 200 mg/L, except for brassicasterol and campestanol
127 where the LDR was 0.1 to 100 mg/L due to the limited of availability of reference standards. The

correlation coefficient for all sterols was >0.99. The LDR for cholesterol was determined to be 0.1 to 1000 mg/L with a correlation coefficient >0.99 (y=0.044x).

2.3 Consumables and equipment

Incubation of the hydrolysis and saponification sample mixtures were performed in a shaking water bath (WB4D, Ratek, Melbourne, Australia) with a maximum temperature of 100˚C. Sample hydrolysis was performed using 44 and 60 mL screwcap vials with Teflon septa. Evaporation of solvents of volumes greater than 1 mL was performed using an evaporating nitrogen manifold (Thermo Fisher, Australia) and derivatization/extract evaporation for volumes less than 1 mL were performed using a heating block with GC holding plate and evaporating manifold (Ratek, Melbourne, Australia).

The SPE cartridges used included a 5 g amino propyl phase cartridge (particle size 40 µm and volume of 20 mL purchased from Agilent Technologies, Melbourne, Australia) and a silica Sep-Pak 690 mg cartridge (particle size 55-105 µm and volume of 2 mL purchased from Waters, Melbourne Australia). The SPE was performed in a vacuum manifold (Sigma Aldrich, Australia) and all small volumes were accurately measured using piston operated volume aspirator ranging from 10 to 1000 µL, or a positive displacement piston operated volume aspirator.

2.4 GC analysis

Derivatized sample extracts were analyzed using an Agilent 7890 Gas Chromatograph coupled with a 5975 C MS and FID detectors. Chromatographic separation was achieved using a HP-5MS (5%-phenyl-methylpolysiloxane 30 m x 0.25 mm, film thickness 0.25 µm) capillary column with a 3 m deactivated silica column (guard column). The GC temperature program for the analysis initially started at 245°C and was ramped to 265°C at 2°C min^{-1}, followed by a ramp to 290°C at 3.5°C min^{-1} where it was maintained for 8 minutes. The carrier gas used was helium with a flow 3.1 mL min^{-1}. A

split injection mode of 1:5 was used with an injector temperature 310°C. The instrumentation was also program to perform a back-flush after each analysis for 7.5 minutes at 24.6 psi. The FID temperature was set at 300°C (total detector flow 30 m min^{-1}) and the MS source at 250°C (scan mode ion 50-500 atomic mass units) with a 1:1 split for each detector.

2.5 Feed sampling and extraction

2.5.1 Feed samples

All feed samples used for this study were provided by the Department of Economic Development, Jobs, Transport and Resources and included lucerne, pasture (rye grass), maize silage, pasture silage, grape marc (dried and ensiled), wheat, canola, tannin, mineral mix, cotton oil and molasses. Replicate samples obtained between 2010 and 2011 from different seasons were also analyzed. All feed samples were stored in the freezer until analysis and were then prepared using a grinding mill (Foss CT 293 Cyclotec™) and a high-powered homogenizer (Robot Coupe Blixer® 3). Total solids were also determined for all cattle feed samples by subtracting the moisture content which was determined gravimetrically by oven drying at 104°C until constant weight was obtained.

2.5.2 Extraction of cattle feed samples

For each sample, 0.5 -1.5 g was weighed into a 60 mL screw cap vial containing 2-3 boiling chips. A 5 mL aliquot of heptane, known amounts of 5β-cholestan-3α-ol and 10 mL of 4 M aqueous HCl was added to the sample vial which was then capped, mixed and incubated at 80°C for 30 minutes. The vial was shaken intermittently every 10-15 minutes during incubation. Following incubation, the vial mixture was cooled to room temperature, then 20 mL of 5 M ethanolic KOH was slowly added to the sample vial in order to neutralize the acid hydrolysis solution. The vial was then recapped, mixed and incubated at 80°C for 30 minutes with intermittent shaking every 10-15 minutes. Following this second incubation, the sample vial was cooled to room temperature before 8 mL of

aqueous 4 M hydrochloric acid and 8 mL of water was added. The vial was recapped, shaken and allowed to settle to form two distinct layers after which the organic layer was transferred to a test tube and the volume reduced to 4 mL using nitrogen gas. Since sterols are non-volatile compounds, they are stable during this evaporation process and are not removed by the nitrogen purge. This step was then followed by sample clean-up using SPE.

2.5.3 Sample clean-up

Prior to sample clean-up, the amino propyl SPE cartridge was conditioned with 15 mL of heptane. Then 1 mL of sample extract was loaded onto the cartridge and allowed to pass through. Another 15 mL of heptane was passed through the cartridge with this fraction discarded. The sterols were then eluted using 25 mL of and 80:20% (v/v) methanol:chloroform mixture, blown down and then transferred to a GC vial for derivatization.

2.5.4 Extraction of unfortified milk

For each milk sample, 5 mL was weighed into a 60 mL screw cap vial containing 2-3 boiling chips. A 5-mL aliquot of heptane, known amounts of 5β-cholestan-3α-ol and 5 mL of 8 M ethanolic HCl was added to the sample vial which was then capped, mixed and incubated at 80°C for 30 minutes. The vial was shaken intermittently every 10-15 minutes during incubation. Following incubation, the vial mixture was cooled to room temperature, then 20 mL of 5 M ethanolic KOH was slowly added to the sample vial. The vial was then recapped, mixed and incubated at 80°C for 30 minutes with intermittent shaking every 10-15 minutes. Following this second incubation, the sample vial was cooled to room temperature before 4 mL of aqueous was added. The vial was recapped, shaken and allowed to settle to form two distinct layers after which the organic layer was transferred to a test tube and the volume reduced to 1 mL under nitrogen gas to produce the sample extract which was then subjected to derivatization.

2.5.5 Sample derivatization

Sample extracts were evaporated to dryness under nitrogen in the GC vial, after which 300 µL of BSTFA+1%TCMS and 700 µL of a 3:4 volume ratio of toluene:pyridine mixture was added. The vial was then capped, shaken and incubated at 80°C for 20 minutes. For samples with insignificant or no phytosterol glycoside content, i.e. high sterol ester content samples, the extraction method presented in "extraction of unfortified milk" was used with some modifications. In the first incubation step, 5 mL of 5 M ethanolic KOH was added to sample vial rather than aqueous HCl. After incubation, the vial was cooled to room temperature and 4 mL of water was added. The vial was then recapped, shaken and allowed to settle until two distinct layers were observed. The organic layer was collected and reduced to 1 mL using nitrogen. The extract was then transferred to a GC vial and the derivatization process as "sample derivatization" was followed.

2.6 Method validation

Method validation was performed using certified reference materials (CRMs) obtained from National Institute of Standard and Technology (NIST, USA) in order to obtain samples where the target sterols were present homogenously throughout the matrices. The CRMs used included NIST 3250 *Serenoa repens* seed fruit (certified for β-sitosterol, stigmasterol and campesterol), and meat homogenate NIST 1546 (certified for cholesterol). The secondary reference material (SRM) studied was Vega pure E, a phytosterol fatty ester paste (BASF^{TM}, certified for β-sitosterol, campesterol, stigmasterol, brassicasterol and stigmastanol). In addition, recoveries were performed on milk samples that were purchased from local markets to investigate whether the method was suitable for low level phytosterol measurements.

2.7 Statistical analysis

The measurement uncertainty (MU) determined for this research was based on ISO/IEC Guide 98-3, 2008 using the top-down approach to incorporate validation data including recovery, duplicates, reference standard calibration uncertainty and quality control for the final uncertainty estimation. This approach was chosen as it would allow for the precision, accuracy and any systematic bias in the methods [38]. For this study, the recovery and duplicate data included 7 milk matrix spikes, 3 water matrix spikes, 9 NIST 1546 meat homogenate CRM samples, 7 NIST 3250 *Serenoa repens* CRM samples, and 26 Vega pure E SRM samples. Given the high cost of the CRM and SRM materials, it was not possible to perform spike-recovery experiments with hay and silage samples. The uncertainty was determined by the square root of the sum of the relative standard deviations of: the standard preparation, the calibration standard, the recovery, and the duplicates. A coverage factor of 2 was applied to the uncertainty to expand the confidence interval to 95%. In addition, an analysis of variance was performed to determine significances between parameters during the method development with a post-hoc analysis using the Tukey approach.

3 Results and discussion

In our previous study, a method for the determination of total sterols was reported [36] which was optimized based on existing saponification methods [8, 39, 40]. In the present study, further optimization of the method was undertaken with the focus on the acid hydrolysis of samples prior to GC analysis. The acid hydrolysis method development comprised of an investigation of critical aspects including: hydrolysis solution composition; acid concentration and incubation period; and sample extract clean-up.

3.1 Effect of ethanol content in acid hydrolysis solution

A total of 13 different feed sample matrices were investigated which included 12 types of animal feed and 1 milk sample. To maximize the extraction of phytosterols from these matrices, the

242 acid hydrolysis solution composition is a critical factor as it contributes to sample solubility and cell
243 lysis of plant materials. Previous studies have utilized two distinct acid hydrolysis solutions prepared
244 in either water (for food samples) [8, 26] or in ethanol (for plant materials) [41]. Given the broad
245 range of matrices in the present study, a hydrolysis solution composition that was suitable for all the
246 matrices was required. Four solution systems were investigated including acid prepared in: water
247 only; 20% v/v ethanol in water; 50 v/v ethanol in water; and ethanol only. For optimization, milk
248 powder, lucerne and Vega pure E (a reference fatty paste) were selected to represent the dominant
249 sample types, i.e. milk, plant and fatty samples respectively.

250 Figure 1 shows the quantity of total sterols extracted from the three sample types using the
251 different hydrolysis solution compositions. The data demonstrates that for fatty samples (i.e. Vega
252 pure E), an increase in ethanol content improved sterol recovery whereas higher water contents
253 decreased the solubility for the subsequent saponification reaction. In the case of lucerne, higher
254 ethanol contents in the hydrolysis solution decreased the total sterol recovery with up to 34% lower
255 sterol recovery in ethanol only compared to a water only acid solution. The recovery of total sterols
256 from milk powder was satisfactory at levels up to and including 50% ethanol. It was therefore
257 concluded that for milk and high fat samples, an acid hydrolysis solution prepared using 50% ethanol
258 in water was adequate. For plant-based samples, an aqueous acid hydrolysis solution was the most
259 suitable.

260 **3.2 Effect of acid concentration and incubation time**

261 Acid concentration is critical to efficiently break the glycosidic bonds *via* hydrolysis in order to
262 maximize the liberation of sterol glycosides. In this study, HCl concentrations of 4, 5, and 6 M were
263 trialed with results showing that no significant recoveries were gained of the three selected matrices
264 using higher acid concentration as shown in Table 1. The hydrolysis incubation time was also studied

265 in order to optimize the time needed to ensure all glycosidic bonds are cleaved. Although the
266 saponification incubation time has been investigated previously [36], the present study further
267 explored the hydrolysis time prior to the addition to saponification mixture to ensure that the
268 hydrolysis solution did not affect the saponification process. Three incubation time brackets were
269 selected, i.e. 30, 60 and 90 minutes, and were applied to the hydrolysis of the three representative
270 samples at 4 M. As shown in Table 1, the results demonstrate that complete hydrolysis was obtained
271 with the minimum incubation period of 30 minutes with no significant increase when incubated for
272 longer times.

273 It was also observed that doubling the volume of the saponification solution after hydrolysis
274 neutralized the acid with any excess continuing the saponification reaction. Further verification with
275 different feed matrices was required to ensure the saponification incubation time of 30 minutes was
276 still applicable from previously optimized conditions [36]. Figure 2 demonstrates that 30 minutes of
277 saponification incubation time was still applicable and no significant gain was obtained for longer
278 incubation periods. Although grape marc gained *ca*. 7% of total sterol recovery, this was not deemed
279 to be significant to increase the incubation time.

280 **3.3 Effect of acid addition following saponification**

281 The composition of plant matrices is generally more complex than milk or fat samples and can
282 therefore require additional or modified extraction techniques. In the sterol extraction method, acid
283 and water are added to the sample mixture after saponification with the addition of water facilitating
284 the solubility of salts, glycerine, and fatty acid salts, while leaving the un-saponifiable fraction to be
285 extracted into the organic phase. The addition of acid neutralized the alkaline saponification solution
286 thereby increasing the ionic strength of the aqueous phase in order to minimize the emulsion
287 between the organic and aqueous layers. This process was very effective for plant samples, however

288 for the milk and fat samples, an emulsion between layers was not observed. In this case, the addition
289 of the acid would potentially result in the conversion of fats to their alcohol conjugate and
290 subsequent solubilization of these compounds into the organic layer thus creating non-targeted
291 interferences.

292 Experiments were performed to determine whether the addition of acid could be omitted for
293 milk and high fat samples only. For the milk and oil sample extracted with and without the addition of
294 acid, the results showed no significant gain was obtained for the addition of acid. The addition of acid
295 after saponification was therefore omitted for milk, high fat, oil or fat only samples. Conversely, the
296 addition of the acid in plant materials was continued in order to optimize the extraction. This was
297 demonstrated by performing a recovery with no acid and no SPE clean-up which resulted in a
298 cholesterol level of 11.5 mg/100 mL of milk. No significant gain was achieved with the use of acid and
299 SPE clean-up with only 11.6 mg/100 mL of cholesterol measured suggesting the additional steps could
300 be omitted without detriment to the recovery.

301 **3.4 Optimization of sample clean-up and elution**

302 The use of SPE was further investigated as a clean-up step in this study as sterols can be
303 selectively purified providing the correct phase is used [42, 43]. Several stationary phases are used
304 for this purpose, including cartridges comprised of silica and an aminopropyl phase [6, 26, 44, 45]. For
305 this study, both silica and aminopropyl solid phase cartridges were selected for clean-up investigation.
306 The initial work was performed using reference standards, with both phases able to perform
307 satisfactory recoveries for the Vega pure E ranging from 90-110% for both silica and aminopropyl
308 phase. However, when sample extracts were trialed, no profile change was observed for the silica SPE
309 and it was subsequently confirmed using GC-MS/FID that the silica cartridge was unable to remove
310 non-targeted compounds from the extracts (data not shown). Conversely, the aminopropyl cartridge

311 was able to significantly remove non-targeted compounds while recovering sterols within a
312 satisfactory range (80-120%) for the level of the component measured in accordance with relevant
313 validation guidelines [46].

314 Shown in Figure 3 (a) and (b) are examples of the lucerne extract chromatograms using the silica
315 and aminopropyl phase in the SPE clean-up, respectively. It is clear that the aminopropyl phase is
316 able to selectively remove non-targeted compounds within the sterol chromatographical range
317 between 13 and 17 minutes, whereas the silica is unable to remove non-sterol compounds from the
318 extracts. The aminopropyl SPE clearly demonstrates greater efficiency at removing non-target
319 compounds eluting at 14 minutes which was identified as the surrogate standard 5βcholestan-3α-ol
320 based on the retention time from the reference standard and mass spectral database library. It was
321 observed that for fractions collected by SPE, not all phytosterols were recovered suggesting that the
322 SPE sorbent capacity was too low to retain the target analyte in the extract. A dilution of the lucerne
323 extracts was therefore trialed on the SPE to determine the phase capacity required for methods
324 extracts. Using 320 mg sorbent, a 1 in 5 dilution resulted in recoveries of 102% and 116% of the
325 surrogate (5β-cholestan-3α-ol) and β-sitosterol respectively. For a 1 in 2 dilution, recoveries of 23%
326 and 38% were obtained for the surrogate and β-sitosterol respectively suggesting that the 1 in 5
327 dilution resulted in more satisfactory recoveries.

328 For the majority of feed matrices, 1.5 g of sorbent in the SPE stage is typically used effectively.
329 However, in this study 5 g of sorbent was used in order to safeguard against possible SPE overload
330 capacity issues for unknown cattle feed matrices. This increase in sorbent material subsequently
331 required the use of additional solvent to discard non-targeted compounds and elute target sterols. As
332 a result of this increase in sorbent, an investigation into appropriate solvent polarity strength was
333 studied to obtain adequate separation between the target and non-target analytes. In this case, the

elution of sterols from the grape marc extract was investigated using 45 mL of chloroform and 25 mL of an 80:20% (v/v) chloroform:methanol mixture. For the chloroform only extraction, 88% and 93% of β-sitosterol and stigmastanol were recovered respectively. For the lower volume mixed solvent, recoveries of 107% and 104% of β-sitosterol and stigmastanol were obtained respectively. The results demonstrate that a reduced volume of the mixed solvent results in a higher recovery than the larger volume of chloroform only which may be due to the greater polarity of the chloroform methanol mixture. It is important to note that during the process of the method development, hazardous and potentially carcinogenic reagents and solvent including toluene, pyridine and chloroform were used sparingly to reduce exposure and to limit the generation toxic waste.

3.5 Method validation

Validation of the total phytosterol and cholesterol extraction methods were performed on two CRMs, one SRM, and additional spiked samples using stigmasterol at the limit of reporting (LOR), 2×LOR, and 5×LOR where the LOR is 0.02 mg/100 mL for milk and 1 mg/100 mL for other liquid samples. In the case of the spiked samples, the spiking was performed prior to the hydrolysis process. As shown in Table 2, the relative standard deviation for all samples was less than 12% and satisfactory recoveries were obtained for the CRM and SRM samples ranging from 80 to 120% as per the Australian Pesticides and Veterinary Medicines Authority guidelines [46]. Total phytosterol content was calculated based on the summation of the identified sterols (i.e. the sum of campesterol, brassicasterol, campestanol, stigmasterol, stigmastanol and β-sitosterol levels). The average recoveries for the spiked milk samples were *ca*. 64, 69 and 80% for the 1×, 2× and 5× LOR samples respectively. This is considered to be satisfactory and the results are as expected for such low-level determinations. The average process recoveries for spiked samples in water were *ca*. 99, 103, and 96% for the respective LOR spikes and this indicates the lower recovery from the spiked milk samples

was due to matrix interference as shown in Table 3. Overall the MU determined for this method for milk at the 1×LOR value of 0.02 mg/100 mL for the individual sterols was ±35% and for the animal feed at the 1×LOR value of 5.0 mg/100 g, the MU determined was ±15% for results falling within the mid-calibration range. In both cases, a 95% confidence interval was used with a coverage factor of 2. The validation data demonstrated that the method is suitable for the analysis of animal feed and milk for both trace and high-level sterol analysis.

3.6 Analysis of cattle feed

Using the validated method, the total sterol levels in a broad range of animal feeds were analyzed. Three separate samples of each feed type were analyzed with the exception of tannin, molasses and cotton seed oil where a single sample from each was analyzed. The average results of the total sterol contents from each sample are presented in Figure 4. The main sterols found in the feed samples were β-sitosterol, stigmasterol, stigmastanol and campesterol. Overall, the highest total sterol content was found in cotton seed oil and the lowest in tannin, although these were among the test feeds comprised of a single sample only. It was also observed that in both the maize and pasture samples that the sterol content was higher in the silage form. Comparison between literature value of total phytosterols was only possible for the wheat and cotton seed oil as, the rest of the animal feed matrices was not available for direct comparison. The total phytosterols in the wheat sample was comparable to values cited by Ruibal-Mendieta*, et al.* [45], however for cotton seed oil it was 40-50% lower than the values reported by Gül and Amar [47]. The differences between the latter literature reference and that of the present study for cotton seed oil may be due to differences in cotton varieties, seasonal variations, or the age of the samples. The general standard deviation within each feed matrix over different subsamples was ≤25% and given that the subsamples were collected over a course of two years, the results demonstrate that the total sterol content in each matrix was

comparable. This suggests that an average result of each matrix can be used to simplify and predict trends associated with feed and sterol content in milk for future studies.

4 Concluding remarks

A method using both acid hydrolysis and saponification was developed in this study to measure total phytosterol levels and was found to be suitable for the analysis of milk and animal feed at trace and naturally occurring levels. An aminopropyl SPE cartridge was found to effectively remove non-targeted analytes from the extract minimizing interference. The method was used to analyse a variety of animal feed types and the highest and lowest total sterol contents were found in cotton seed oil (256 mg/100 g) and tannin (<30 mg/100 g) respectively. Silage samples of pasture and maize had higher sterol content compared to their non-silage counterparts. The average standard deviations for total sterol levels between subsamples with the same feed matrices were ≤25% suggesting a relativity insignificant variation between subsamples. It is therefore recommended that average values can be used to compare trends in sterol contents in milk in future animal feed studies.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure Captions

Figure 1. Total sterols extracted from various sample types with different ethanol contents in the acid hydrolysis solution (error bars represent standard deviation between results from the same batch). Notes: (*a) indicates significances within the group for the acid medium with *p* values <0.5; (*) significant differences were found when compared only to the control (water) within the group.

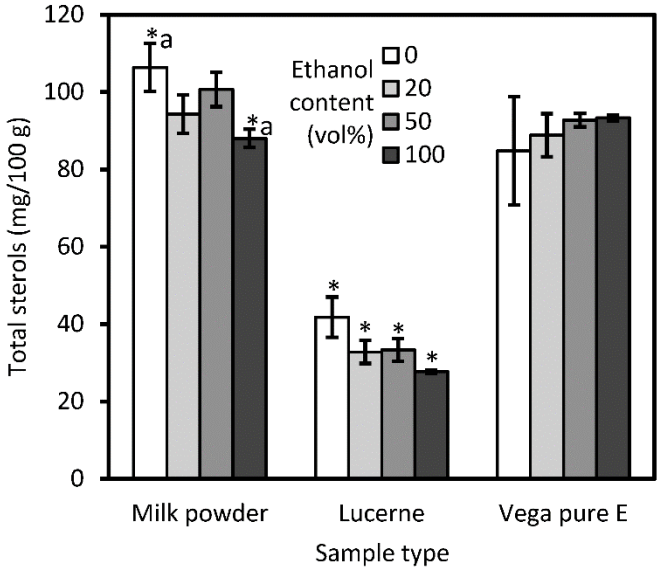

Figure 2. Effect of saponification incubation time on sterol recovery of various feeds. Note: (*) indicates significant differences between data points with p value <0.05.

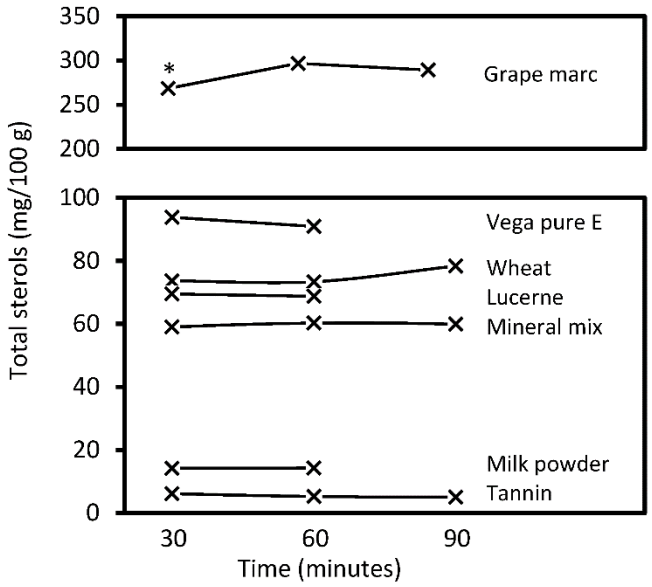

Figure 3. Chromatogram of lucerne extract using (a) silica SPE cartridge and (b) aminopropyl SPE cartridge.

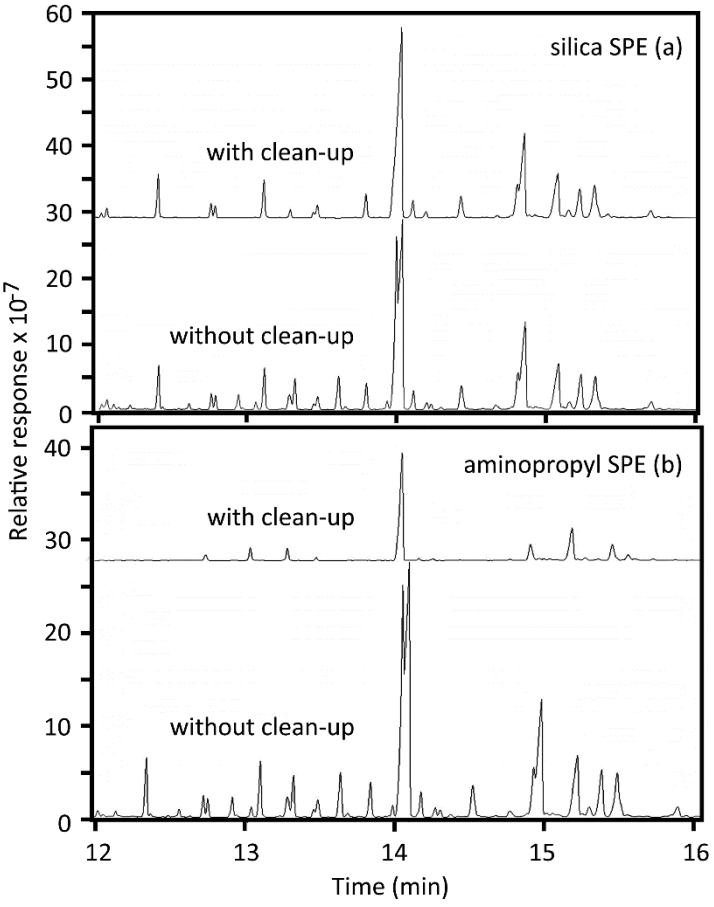

Figure 4. Average total sterols within the same matrices (feed samples marked * were single samples only; error bars represent standard deviation between replicate subsamples). Note the standard deviation within each group was < 25% compared to the overall mean value.